Discussions

Unlike T cell mediated cytotoxicity, target lysis by natural killer (NK) and lymphokine activated killer (LAK) lymphocytes is not class I MHC restricted. Nonetheless, MHC I molecules may influence the NK/LAK effector-target interactions in important ways. Role of class I MHC antigens on NK cells in modulating effector-target interactions was first investigated by Saxena *et al* (107). In this study, class i MHC molecules on effector cells were blocked by using specific polyclonal antisera and the effect of this treatment on NK cell mediated lysis of target cells was studied. Enhanced killing of K562 was observed when anti H-2 alloantibody treated murine NK effector cells were used. Further studies on the mechanism of anti H-2 antibody induced augmentation of NK activity indicated that Fc portion of the alloantibody was crucial for this effect and the effect was seen with cell lines having Fc receptors for IgG (108). Authors suggested that the effector reactive alloantibody modified the interaction between the NK effector and target cells by forming a reverse antibody bridge between effector and target cells which facilitated the killing reaction. The phenomenon was termed as reverse ADCC (R-ADCC) reaction (109). These results were confirmed by Brunda and Herberman (13), who in addition demonstrated the alloantibody induced NK killing even with YAC target cells which lack Fc receptors. In retrospect, these results suggest a role of effector class I MHC molecules in modulating NK effector-target interaction, not only by R-ADCC mechanism, but possibly by additional mechanisms requiring functional involvement of class I MHC molecules on effector cells.

A crucial role for class I MHC antigens on target tumor cells in determining their NK susceptibility was first suggested by Karre *et al* (49). These authors argued that agents or experimental manipulations which result in increased class I MHC antigen expression on tumor cells, also rendered them relatively resistant to NK cells (49,66). This idea found support in several studies where an inverse correlation between NK susceptibility and levels

of class I MHC antigens on target cells, was found (20,49,72,105,106,110). Since cytotoxic T cell mediated tumor cell lysis required the presence of class I MHC antigens on target cells, it was possible that tumor cells , may attempt to escape T cell mediated immune surveillance by down regulating their class I MHC expression. Karre *et al* argued that tumor cells which downregulate their MHC I levels, would become susceptible and get lysed by NK cells. This was indeed an attractive hypothesis since it provided an important raison d'etre for NK cells. Moreover, MHC I negative variants of tumor cells are not uncommon (49,64,127). Even in certain viral infections, where cytotoxic T cell surveillance is most crucial, attempts by the virus to down regulate surface class I MHC molecules on host cells, is well documented (19,100).

Soon after the hypothesis of inverse correlation between NK susceptibility and class I MHC antigen levels was propounded, several groups reported data, where such inverse correlation was not observed (21,31,85,92,139). Attempts have been made to explain the apparent lack of effect of alterations in class I MHC antigen levels on NK susceptibility of some tumor cell lines (65,66,105). In systems where the inverse correlation works, two hypotheses have been proposed to explain the mechanisms by which class I MHC molecules may lower the target cell killing by NK cells. These are, (a) Inhibitory signal hypothesis: According to this hypothesis, class I MHC molecules on target cells can send a negative downregulatory signal to NK effector cells, thereby protecting the target cells from lysis and (b) Interference hypothesis: This hypothesis states that class I MHC molecules on target cells can interfere with NK recognition of target structures on tumor cells. Increased levels of class I MHC molecules on target cells would therefore impede NK-target interactions resulting in lower target lysis.

LAK cells are essentially derived by IL-2 activation of NK cells, and quantitatively, target lysis is significantly greater when LAK effector cells are used. In addition most tumor cell

lines, even if they are NK resistant, are effectively lysed by LAK cells. These reasons make LAK system convenient for studying the role of class I MHC antigens in regulating target cell susceptibility. In the present study, we have used five murine and five human tumor cell lines as panels of targets and besides looking at the direct effects of class I MHC antigens on the LAK susceptibility of these tumor cells, we have also examined changes brought about by modulation of class I MHC levels on the ability of tumor cells to competitively inhibit the lysis of other tumor target cells in their respective panels. Results obtained with the mouse and human panels of targets will be discussed separately.

Mouse Study: Basic LAK susceptibility of the target cell lines were in the order, P815 > YAC > SP20 > EL4 > L929. Levels of class I MHC antigens on these tumor cell lines were in the order P815 > SP20 > L929 > YAC > EL4. Basal LAK susceptibilities and basal MHC I expressions therefore do not appear to be inversely correlated. We reasoned that LAK effector cells may "sense" the density of class I MHC expression on the target cells rather than the levels of expression on the whole cell. The different tumor cells used had different cell sizes as evident from the flowcytometric data on forward scatters for these cell lines. When the levels of class I MHC antigens were normalized to get relative densities of class I MHC molecules on cell surfaces of different tumor cell lines, the order of expression remained essentially unaltered. Thus basal susceptibilities of tumor cell lines were not inversely correlated with either total cellular levels of class I MHC antigens or their density of expression on tumor cells. IFN-g treatment enhanced the expression of MHC class I antigens in all cases and a concomitant decline in LAK susceptibility was noticed for all cell lines except EL4. Treatment with acid pH (3.0) buffer lowered the expression of class I MHC antigens on all tumor cells but an increase in LAK susceptibility was seen with P815, SP20 and L929 cell lines only.

For discussion sake, we have expressed the results of our

competition experiments with untreated competitor cells, in the form of a shade diagram, where all combinations of target and competitor cells have been represented and the darkness of the shade for a given combination is proportional to the competition efficacy observed in that combination (Fig. 34). Within the larger square ABCD, two squares AEFG (representing all combinations of YAC, P815 and SP20 cells) and FJCK (representing combinations of EL4 and L929) stand out as significantly darker sub squares. These results indicate that there could exist two groups of target cells within the five tumor cell lines used. P815, YAC and SP20 constituted one group where all the three lines could efficiently inhibit the lysis of each other in cold target competition assays, indicating that these tumor cell lines could share common target structures. For the same reason, L929 and EL4 cells constituted the other group. Cells from one group did not inhibit the lysis of cells from the other group in competition assays, with the sole exception of L929 inhibition of YAC lysis. Enhanced class I MHC antigen levels on EL4 cells did not alter their susceptibility to LAK lysis. This had previously led us to suggest that EL4 cells could be lysed by a distinct LAK cell subpopulation, insensitive to target MHC I antigen levels (105). Our current results support the possibility that two distinct LAK cell subpopulations may be responsible for the lysis of the two groups of tumor cells. LAK cells lysing EL4 targets however, may also lyse L929 target cells and as an inverse correlation existed between the class I MHC antigen levels on L929 cells and their LAK susceptibility, this LAK subset may not be universally insensitive to changes in MHC I antigen levels on target cells.

Competition can be concieved as an engagement of effector cells by the competitor cells so as to distract the former away from the target cells. If MHC class I antigens send a down regulatory signal to the effector cells (first hypothesis, see above) enhanced class I MHC antigens on IFN-g treated competitors should send a negative signal to the effector cells resulting in a decline in the observed target lysis. Even if one argues that a

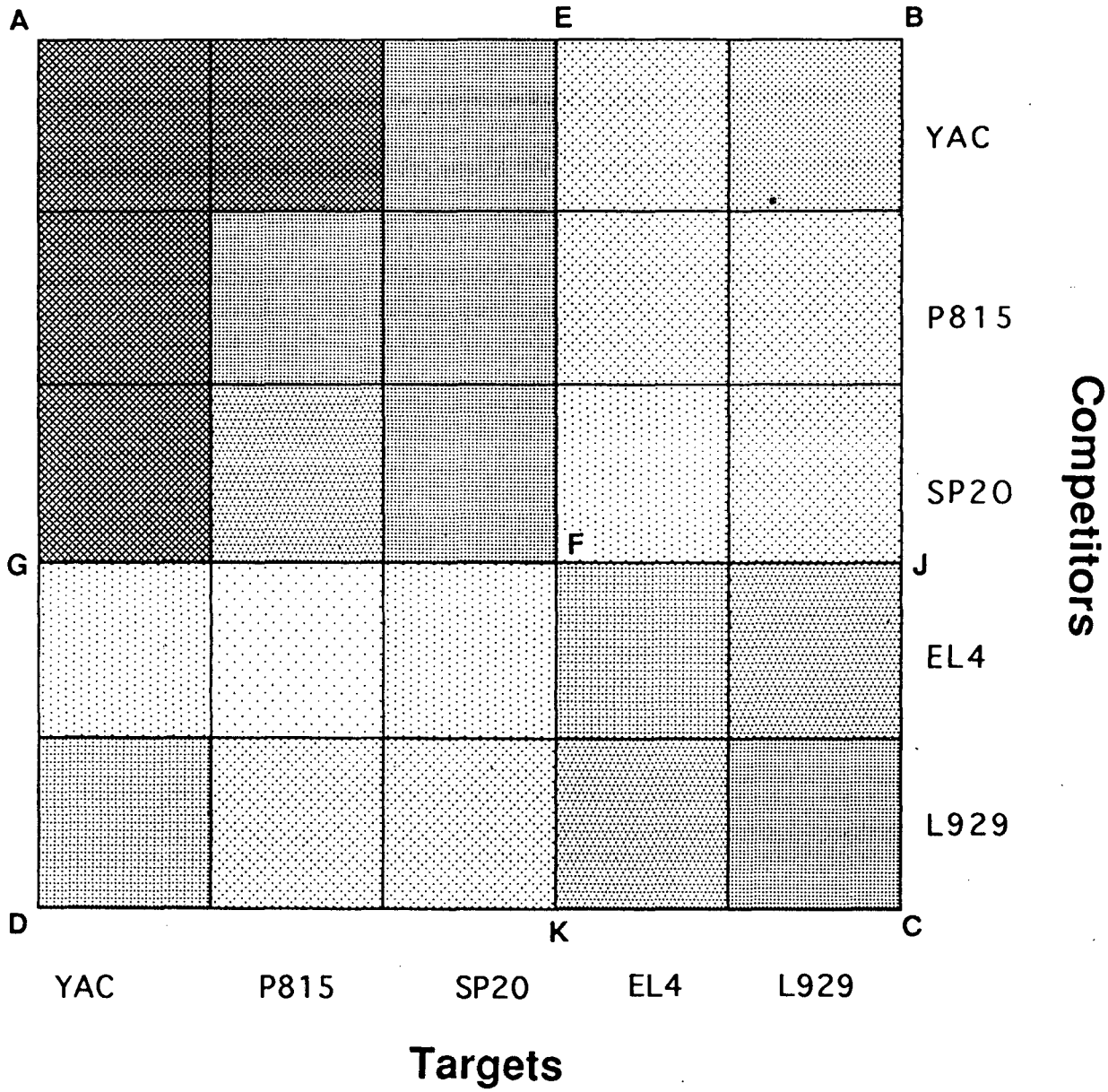
A
E
B
YAC
P815
SP20
Competitors
G
F
J
EL4
L929
D
K
C
YAC
P815
SP20
EL4
L929
Targets

Fig. 34

target cell expressing enhanced class I MHC antigen levels, sends a down regulatory signal only for itself and not for the bystander cells, control and IFN-g treated competitors should have competed with equal efficiencies in our experiments. Our results however, clearly indicate that for group I tumor cells (P815, YAC and SP20) the competition ability of tumor cells declined significantly as a result of IFN-g treatment. Our results, thus do not support the hypothesis of a inhibitory signal to the effector cells by target class I MHC molecules, at least for group I tumor cells. In group II targets (EL4 and L929), IFN-g treatment lowered the ability of L929 cells to compete with EL4 cells. Interestingly, the competing ability of EL4 remained unaltered by IFN-g treatment, which should be viewed along with the fact that (a) EL4 has the lowest basal levels of class I MHC antigens, which remains relatively the lowest even after IFN-g treatment, and (b) LAK susceptibility of EL4 cells was also not influenced by IFN-g treatment. It is possible that IFN-g induced changes in class I MHC antigen levels on EL4 cells may not be sufficient to cause alterations in its LAK susceptibility or competition ability.

The second hypothesis of interference in effector - target interaction by class I MHC antigens, can explain our results, specially for group I targets. Higher class I MHC antigens on competitors would render them less effective in engaging the effector cells, freeing them to interact more with the target cells, resulting in greater target lysis.

Results obtained with pH 3.0 exposed target cells, do not fit with the views on the effect of class I MHC antigens on competition ability of tumor cells. Even though there was a significant decline in the expression of class I MHC antigens as a result of pH 3.0 treatment, enhanced LAK susceptibility was seen only with P815, SP20 and L929 cell lines. In addition, competition efficacy of all tumor cell lines remained unaffected by pH 3.0 treatment. If class I MHC antigen levels solely determined the LAK susceptibility and competition efficacy of tumor cells, these discrepancies would not be expected. It should be noted here that

IFN-g treatment was given over 48 hours in which a variety of other cellular processes would have been influenced since IFN is known to be a pleiotropic cytokine (22). Acid pH treatment on the other hand, was a quick 2 minutes exposure, which selectively denatured class I MHC antigens. Changes in LAK susceptibility and competition efficiencies of tumor cells seen as a result of IFN-g treatment could be due to attendant cellular changes accompanying the enhanced expression of class I MHC antigens. It is also possible that pH 3.0 treatment renders some of the tumor cells more sensitive to lytic process, at a level subsequent to the recognition event. Competition efficacy depends upon sharing of target structures and overall initial physical interaction of effector cells with competitor cells, and pH 3.0 treatment may leave this initial process unaffected.

Human Study: Five human tumor cell lines (K562, MOLT-4, Raji, Daudi and HR7) were used in this study. K562 and Daudi cell lines had no basal expression of class I MHC antigens. Raji and HR7 cells had the highest levels, whereas MOLT-4 had relatively lower levels of these antigens. As discussed for the murine system, expressing the levels of class I MHC antigens in terms of densities of MHC I molecules, did not alter the order of expression of class I MHC antigens on human tumor cell lines. Order of LAK susceptibilities of these tumor cells was K562 > MOLT-4 > Raji > Daudi > HR7. This order of LAK susceptibility seems to be reverse of the order of basal MHC I levels on these tumor cells, with the exception of Daudi cells, which expressed no class I MHC antigens, but still had a relatively low LAK susceptibility.

Percent increase in class I MHC expression on IFN-g treated K562 was the highest, but in absolute terms, they still expressed very low levels of class I MHC antigens as compared to the other cell lines. Yet, the effect of IFN-g treatment on LAK susceptibility was maximum in K562 cells. Interestingly, Raji cells did not increase their expression of class I MHC antigens,

but their LAK susceptibility still declined significantly in response to IFN-g. MOLT-4 cells on the other hand showed an increased class I MHC antigen expression in response to IFN-g, but no concomitant decline in LAK susceptibility was seen. Daudi cells neither showed an increase in class I MHC antigen expression, nor a decline in LAK susceptibility, in response to IFN-g. Inverse relationship between the class I MHC antigen levels and LAK susceptibility was clearly seen for HR7 cells. Taken together, these results do not seem to lend an unqualified support to the hypothesis of inverse correlation between the tumor class I MHC levels and their LAK susceptibility.

Our competition experiments have two components. Firstly, the specificity of competition and secondly the effect of IFN-g on the performance of competitor cells. Different patterns were seen with regard to specificity of competition. A shade diagram depicting the efficacies of competition in different combinations of target and competitor cells, is shown in Fig. 35. Unlike the case of murine system (Fig. 34), no discrete tumor cell groups could be identified on the basis of competition data. K562 cells could inhibit the lysis of all other targets, but Daudi cells on the other end of the spectrum, could competitively inhibit only their own lysis but not that of any other tumor cell lines. Raji, MOLT-4 and HR7 tumor cells had intermediate specificities. These results can be explained if multiple target structures are postulated on different tumor cell lines. Thus, K562 cells may interact with LAK effector cells through several target structures, whereas Daudi cells may share only one or some of these target structures. This proposition may explain why K562 cells could efficiently inhibit the lysis of Daudi cells but the latter had no inhibitory effect on the lysis of K562 cells by LAK effector cells.

Performance of K562, Raji and HR7 cells in competition assays was significantly depressed by IFN-g treatment. By the arguments used above for the mouse system, hypothesis of negative signal by target class I MHC molecules to effector LAK cells, does not appear to be concurrent with our data of competition experiments.

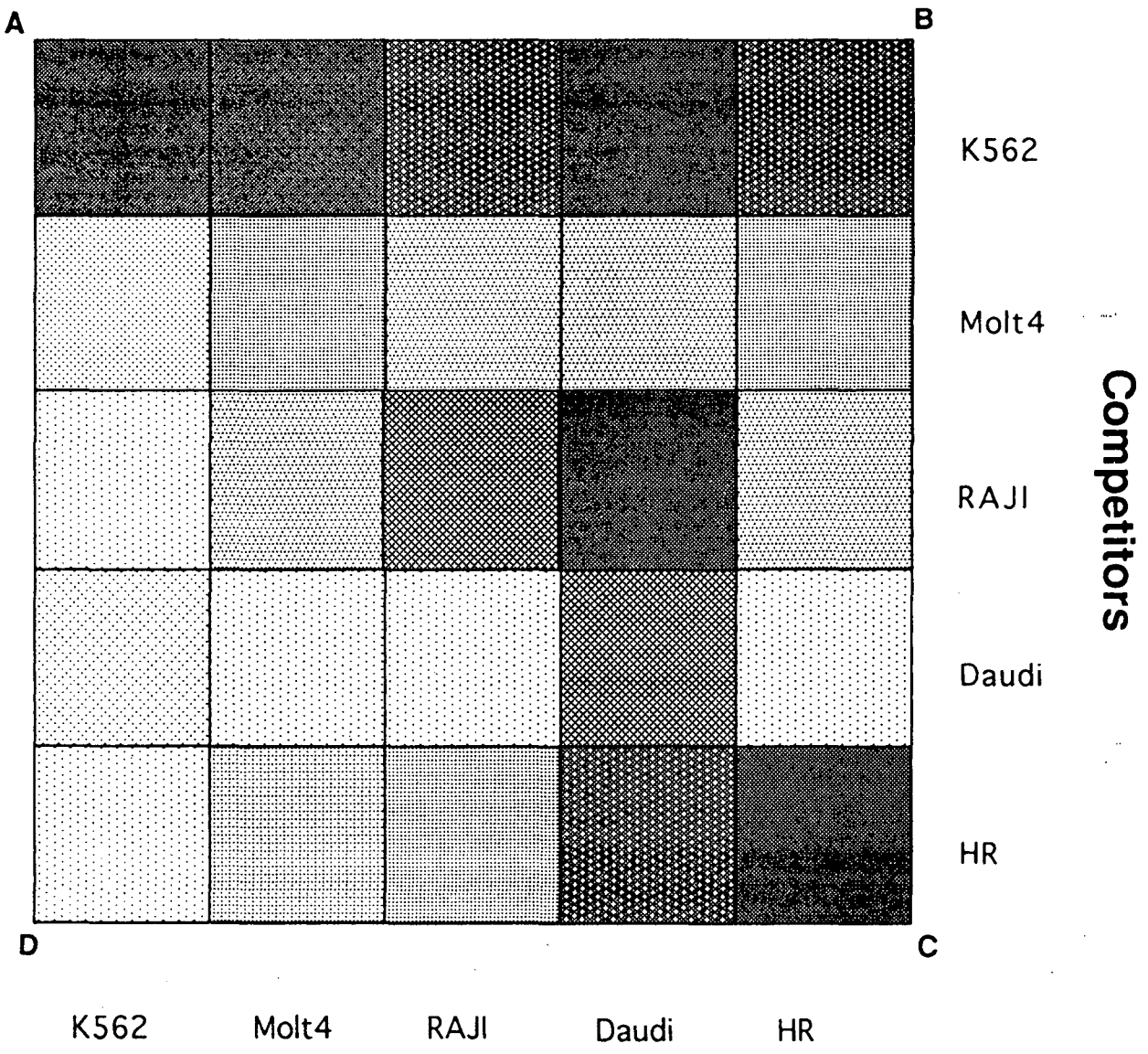
A
B
K562
Molt4
RAJI
Daudi
HR
Competitors
D
C
K562
Molt4
RAJI
Daudi
HR
Targets

Fig. 35

Decreased potency of the IFN-g treated tumor cells with enhanced class I MHC antigen levels, to compete in cold target competition assays can be explained by postulating an interference of effector-target interaction by target class I MHC molecules. Thus competing tumor cells with increased MHC I antigen expression will interact less with effector cells thereby enabling them to interact more with the target cells, resulting in a better target lysis.

Results obtained with pH 3.0 treated tumor cell lines indicate that whereas an increase in LAK susceptibility accompanied an acid induced decrease in class I MHC antigen levels, competition ability of all tumor cells remained unaltered by pH 3.0 treatment. These results are very similar to the ones observed in the murine system. Possible reasons for this lack of effect of pH 3.0 treatment on competition efficacies of tumor cell lines have been discussed above.

Recovery of class I MHC antigen levels and LAK susceptibilities: Even though competition efficacies remained unaltered in response to pH 3.0 treatment, basal LAK susceptibility did increase significantly. Recoveries of class I MHC antigen levels and basal LAK susceptibilities were studied for one mouse (P815) and one human (Raji) tumor cell line. In both the tumor cell lines, MHC I expression went down by 70-80 % and LAK susceptibility simultaneously went up by 3 folds after pH 3.0 treatment. For both target cells LAK susceptibility steadily declined as the MHC I levels started to regenerate on pH 3.0 treated tumor cells and appear to become normal when MHC I expression was only partially restored to 60-70 % of the normal. These results support the inverse correlation of LAK susceptibility and MHC I expression. In a semi-log plot, X-scale is compressed towards the higher side. It follows that relatively smaller absolute changes in MHC I expression towards the lower end of the scale will induce significant changes in LAK susceptibility whereas, towards the higher end of the scale, similar changes in MHC I expression may

induce only marginal changes in LAK susceptibility. Our results thus indicate that even though LAK susceptibility is inversely correlated to target MHC I expression, the effect is more pronounced within lower ranges of MHC I expression. If this conclusion is generalized, one can expect the MHC I expression related changes in LAK susceptibility to be more pronounced in tumor cells with low basal MHC I expression cells and less so for high basal expressors of MHC I. This proposition will however require further validation, using several other tumor cell lines expressing high or low MHC I antigens.

Leukemia Study: In the last part of this study, we wanted to see if the changes in LAK susceptibilities seen with *in vitro* propagated tumor cell lines, also occured with fresh tumor cells. PBLs from leukemia patients were used for this purpose. One problem faced here was that the PBLs from leukemia patients would also have a viable number of normal lymphocytes, and there is no method available to seperate normal and leukemia cells. We have found that while normal PBLs do not survive extended culture durations (about 7-9 days), leukemia cells do survive under these conditions. All leukemia cell preparations were therefore cultured for 9 days in order to reduce the normal PBL contamination which otherwise could potentially interfere in our assays. Our results of this study had a good deal of variations, which are expected in such a system. Overall however, we did find a significant inverse correlation between class I MHC levels on fresh leukemia cells and their LAK susceptibility, when MHC I antigen levels were modulated by either IFN-g treatment or pH 3.0 exposure.

We started this study with an aim to understand the role of target cell class I MHC antigen levels on their interaction with LAK effector cells. While our data does strongly suggest an important role of these antigens in regulating the LAK effector-target interaction, it is also clear from our results that changes in class I MHC levels alone do not constitute a necessary and/or sufficient condition to influence the effector-taregt interaction

or target lysis by the effector cells. Agents which modulate class I MHC antigen levels, may also induce other changes in target cells, which may contribute significantly to overall alterations in LAK effector-target interaction. Such changes are poorly understood at present and must be studied further in order to get a clear picture of the nature of LAK effector-target interactions and mechanisms which modulate their interactions.